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Serum Vpr regulates productive infection and latency of human immunodeficiency virus type 1

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ABSTRACT **In human immunodeficiency virus (HIV)-positive individuals, the vast majority of infected peripheral blood cells and lymph node cells may be latently or nonproductively infected. The *vpr* open reading frame of HIV-1 encodes a 15-kDa virion-associated protein, Vpr. The *vpr* gene has been shown to increase virus replication in T cells and monocyte/macrophages *in vitro*. We have previously reported that *vpr* expression in various tumor lines leads to growth inhibition and differentiation, indicating that Vpr may function as a regulator of cellular permissiveness to HIV replication. Here we show that Vpr protein is present in significant amounts in the serum of AIDS patients. Purified serum Vpr activated virus expression from five latently infected cell lines, U1, OM.10.1, ACH-2, J1.1, and LL58. Serum Vpr also activated virus expression from resting peripheral blood mononuclear cells of HIV-infected individuals. Together, these findings implicate serum Vpr in the activation of HIV replication *in vivo* and in the control of latency. Anti-Vpr antibodies inhibited Vpr activity, suggesting that humoral immunity modulates Vpr activity *in vivo*. These results have broad implications for the virus life cycle and for the prospective control of HIV replication and pathogenesis.**

The persistence of low levels of human immunodeficiency virus (HIV) for years prior to the onset of AIDS is facilitated in part by the ability of the virus to establish latent infection in several tissues, including lymphoid organs (for review, see ref. 1). A high absolute number of cells in the lymphoid tissues are infected throughout the course of HIV infection (2–6), but the virus may be latent in ≥99% of these cells (2, 4). When peripheral blood cells of HIV-infected individuals are cultured *in vitro*, virus production is usually undetectable unless the cells are stimulated with antigen, mitogen, or cytokines (7–11). Activation of HIV expression *in vivo* has been linked to immune activation and cytokine production (reviewed in ref. 12).

Latently infected transformed T-lymphocyte, B-lymphocyte, and promonocytic cell lines have been developed as *in vitro* models for latency (reviewed in refs. 1 and 13). These lines contain integrated provirus but express low levels of viral message and protein (14). HIV expression can be induced by a variety of agents such as phorbol esters and cytokines including tumor necrosis factor α, granulocyte/macrophage-colony-stimulating factor, and interleukins 1 and 6 (15–21). The relative levels of HIV regulatory proteins expressed have also been implicated in the control of latency (reviewed in ref. 1).

Study of the replication of *vpr* mutant viruses has shown that *vpr* increases HIV and simian immunodeficiency virus (SIV) replication in T cells and monocyte/macrophages (22–26). SIV *vpr* may be necessary for the viral life cycle *in vivo* (27). *vpr* inhibits the growth of, and induces differentiation in, muscle tumor lines *in vitro* and inhibits the growth of other tumor lines (28). We propose that Vpr regulates cellular processes linked to HIV replication, particularly in cell types, such as macrophages, in which HIV can replicate in the absence of cell proliferation. We have recently found that recombinant Vpr protein increases virus infection/replication *in vitro* (48).

Vpr is a constituent of the virion (29–31). Since breakdown of HIV particles occurs *in vivo*, this would provide a mechanism to release Vpr into the bodily fluids. Accordingly, we investigated the presence of Vpr in the serum and the cerebrospinal fluid (CSF) of HIV⁺ individuals and found significant levels. We examined the biological activity of serum Vpr with respect to virus replication in latently infected cell lines and peripheral blood mononuclear cells (PBMCs) of HIV⁺ individuals and found that serum Vpr was a powerful activator of HIV expression in each case.

MATERIALS AND METHODS

Cells, Cell Culture, and Serum. Latently infected cell lines were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (NIH ARRRP): T-lymphoid cell lines J1.1 (16) and ACH-2 (20), promonocytic lines OM.10.1 (32) and U1 (21), and the B-lymphoid line LL58 (33). PBMCs were isolated from freshly collected blood by Ficoll/Hypaque centrifugation. All cells were maintained in RPMI 1640 with 10% fetal bovine serum, penicillin/streptomycin, fungizone, 2-mercaptoethanol, and 25 mM Hepes (pH 7.4) in a humidified 5% CO_2 environment at 37°C.

For measurement of protein levels, serum and CSF samples from HIV⁺ individuals were collected in heparinized tubes and stored at −80°C until use.

Vpr Purification. Full-length recombinant HIV-1 Vpr protein was produced in High Five cells (*Trichoplusia ni*) (Invitrogen) following infection with recombinant baculovirus containing the *vpr* open reading frame of HIV-1 NL43. Recombinant Vpr showed identical SDS/PAGE migration to viral and serum Vpr and identical reactivity with anti-Vpr antibodies and HIV⁺ serum in Western blot and ELISA (data not shown).

LR1 rabbit anti-Vpr serum was prepared by immunization of a rabbit with partially purified recombinant Vpr, followed by two immunizations with three Vpr peptides coupled to keyhole limpet hemocyanin (HIV-1 NL43 aa 9–20; GPQREPYNYWTL; 41–55, SLGQHIYETYGDTWA; and 81–96, HFRIGCRSHRIGITRQRRARNGASRS).

Serum from HIV⁺ patients was diluted 1:4 in 10 mM Tris, pH 8.0/100 mM NaCl/0.5% (vol/vol) Triton X-100 with protease inhibitors: aprotinin, leupeptin, and pepstatin A, each at 2 μg/ml; and phenylmethanesulfonyl fluoride at 1 mM. This solution was passed through an LR1-protein G immunoaffinity column, and the column was washed with phosphate-buffered saline (PBS) plus 0.5% Triton X-100. Three bed volumes of pre-elution buffer (10 mM sodium

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Abbreviations: CSF, cerebrospinal fluid; HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate.

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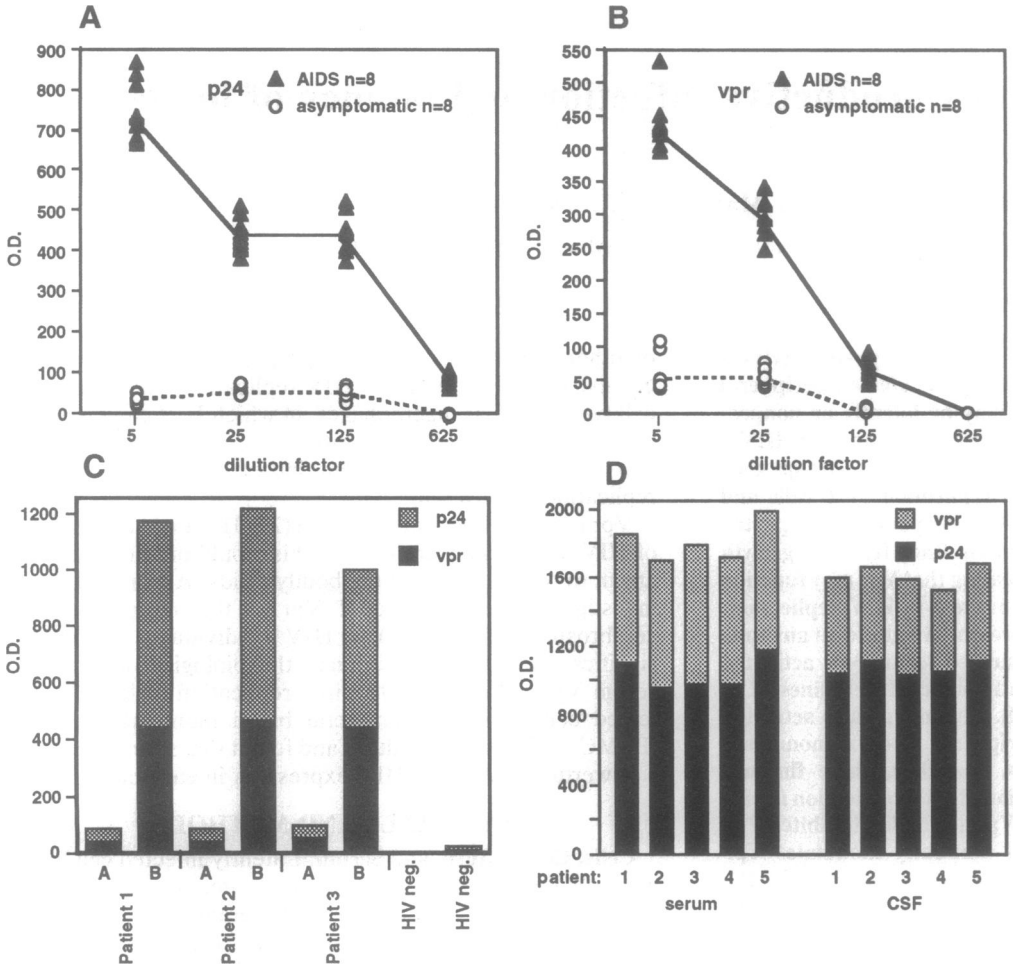

FIG. 1. Vpr protein in the serum and CSF of HIV$^+$ individuals. (*A*) p24 gag protein in the serum of eight asymptomatic HIV$^+$ persons and eight AIDS patients detected at various dilutions. Lines connect median values in each group. (*B*) Vpr protein plotted as in *A*. (*C*) Vpr and p24 in three HIV$^+$ individuals over time during the development of HIV antigenemia in late-stage infection. Patient 1, sample B was collected 16 months after sample A; patient 2, 12 months after; patient 3, 16 months after. Sera from two HIV$^-$ individuals are shown for comparison. All samples were tested at 1:5 dilution. (*D*) Vpr and p24 in the CSF (1:5 dilution) of five HIV$^+$ patients with neurological disorders.

phosphate, pH 8.0/0.5% Triton X-100) was passed through, followed by elution buffer (100 mM triethanolamine, pH 11.5/0.5% Triton X-100). The eluted fractions were collected in 0.05 volume of 1 M sodium phosphate, pH 6.8/0.05% Triton X-100. For use in tissue culture, Vpr-containing fractions were passed through a detergent-removal column (Pierce), dialyzed extensively against PBS, filter sterilized, and stored at 4°C.

ELISA. p24 Gag antigen was measured by capture ELISA using a monoclonal antibody in solid phase (V7.8, obtained from Evan Hersh through the NIH ARRRP) and polyclonal sheep anti-p24 (obtained from Michael Phelan through the NIH ARRRP) followed by a peroxidase-coupled anti-sheep antibody (Boehringer Mannheim) for detection of bound antigen. Commercial recombinant p24 (American Biotechnologies, Cambridge, MA) was titrated as a standard curve and the response was linear on logarithmic plots over the range 2.0–10^4 pg/ml. Sensitivity was 5–10 pg/ml in tissue culture supernatants owing to a dilution of 1:10 used in the assay. Standard deviation in ELISA duplicates was typically <1% to 10%. Standard deviations shown were calculated from duplicated wells of infected cells.

For ELISA of Vpr, rabbit serum recognizing the N terminus of Vpr [aa 2–21; sequence (with added N-terminal cysteine), CEQAPEDQGPQREPHNEWTLE] was obtained from Brian Cullen through the NIH ARRRP (34). This serum was immobilized in wells of a 96-well plate (Immulon II, Dynatech) in 0.2 M carbonate/bicarbonate buffer (pH 9.2). Bound antigen was detected with a mouse anti-Vpr peptide serum (aa 81–86), followed by a peroxidase-coupled anti-mouse antibody (Boehringer Mannheim).

Stimulation of HIV-Infected Cell Lines and PBMCs with Serum Vpr. Serum Vpr was added to wells of cells in 96-well flat-bottom plates with or without antibody overnight (12–14 hr); then the medium was replaced with normal culture medium. Samples were collected daily and fresh medium was added as required to all wells uniformly.

RESULTS

Examination of Vpr Protein in HIV$^+$ Asymptomatic Subjects and HIV$^+$ AIDS Patients. Sera from eight asymptomatic

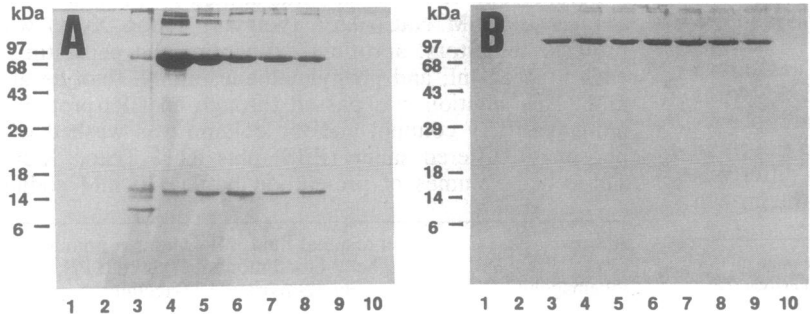

FIG. 2. Purification of Vpr from HIV$^+$ AIDS patient serum. (*A*) Sera from five AIDS patients were pooled and Vpr was purified over an anti-Vpr immunoaffinity column. Ten fractions were collected and analyzed by SDS/15% PAGE followed by silver staining. (*B*) Serum from an HIV$^-$ donor was treated as in *A*.

HIV$^+$ individuals and eight HIV$^+$ AIDS patients were examined for the presence of p24 Gag (Fig. 1*A*) and Vpr (Fig. 1*B*). p24 and Vpr were detectable in each patient, with the level of Vpr present correlating with the degree of p24 antigenemia observed in the two populations. In addition, sera from a further five asymptomatic HIV$^+$ individuals and 47 AIDS patients were examined and the same pattern of Vpr expression was observed (data not shown). Samples from an additional three patients were collected before and during late-stage viremia (Fig. 1*C*). In each patient Vpr increased concomitantly with the rise in detectable p24. Given that Vpr is a constituent of the viral particle, a direct correlation observed between levels of Vpr and another viral protein is not surprising.

Vpr in the CSF of HIV-Infected Individuals. Neurological disorders and dementia are major problems associated with HIV infection and are often the presenting symptoms (35). HIV infects macrophages *in vivo* that may cross the blood–brain barrier, and infection of tissues in immune-privileged areas is thought to occur following entry of HIV-infected macrophages or microglia (36, 37). HIV virus and Gag antigens are common in the CSF of HIV$^+$ individuals with neurological disorders. Therefore, Vpr may also be present in these tissues. CSF from five HIV$^+$ individuals with neurological disease was examined for the presence of Vpr and p24. In each case, levels of Vpr and p24 Gag were similar to the levels detected in serum (Fig. 1*D*).

Purification of Vpr from HIV$^+$ AIDS Patients. The biological activity of Vpr (28) and the presence of Vpr in the tissues of HIV-infected individuals prompted us to examine this protein directly from serum. Serum Vpr was partially purified from AIDS patients by an immunoaffinity column and was examined on a silver-stained SDS/polyacrylamide gel (Fig. 2*A*). Proteins from normal human serum were prepared and analyzed in the same manner (Fig. 2*B*). A band at around 97–105 kDa was observed in both the HIV$^-$ and the HIV$^+$ human serum. This protein did not react with anti-Vpr antibodies in ELISA and so most likely represents protein

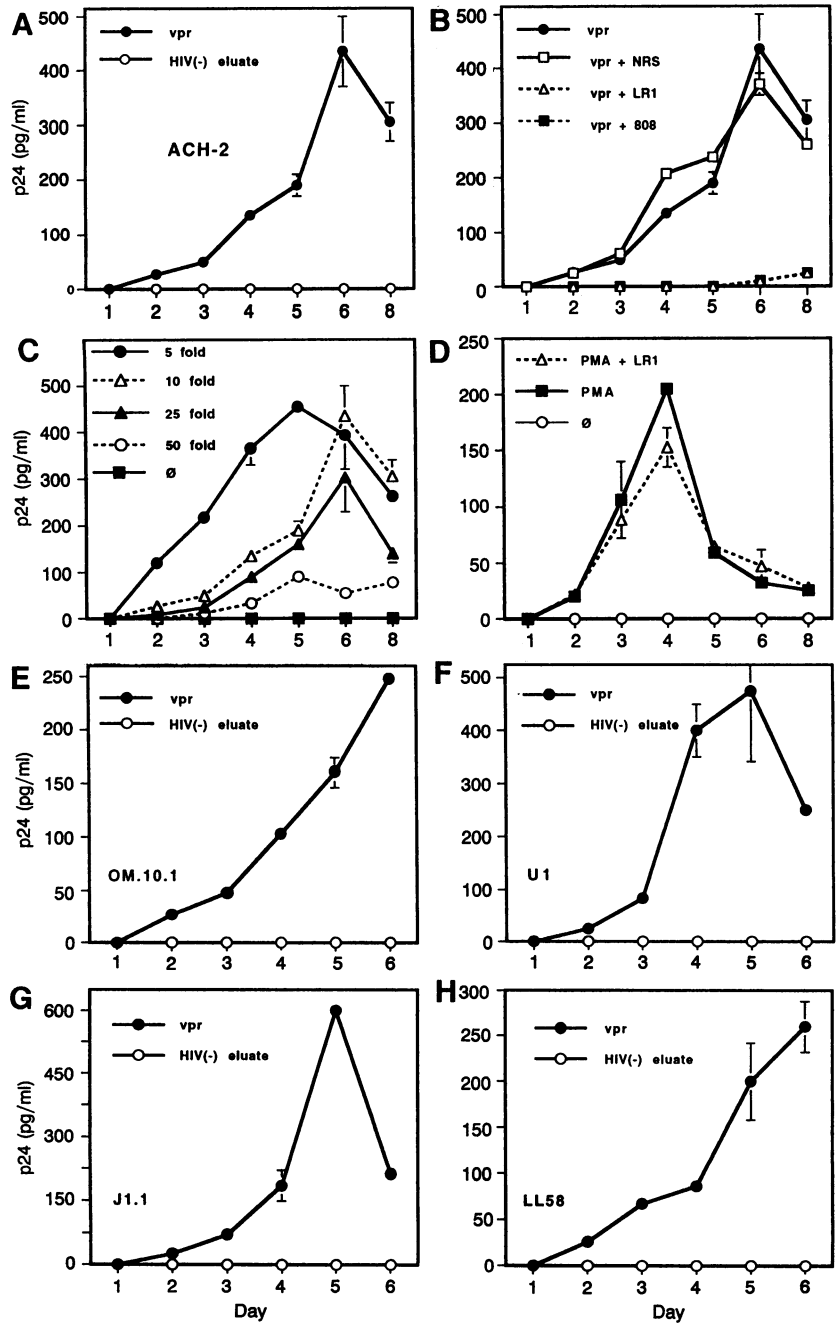

FIG. 3. (*A* and *E–H*) Activation of HIV expression from five latently infected cell lines. Serum Vpr (●) or HIV$^-$ eluate (○) was added at 0.1 (*A*) or 0.2 (*E–H*) times the serum concentration of Vpr to 5 × 10^4 cells on day 1. Vpr specificity is demonstrated by the failure of HIV$^-$ serum eluate (○) to activate HIV expression in each cell line. (*B*) Inhibition of Vpr activity by anti-Vpr antibodies but not by nonimmune rabbit serum (NRS). The 808 anti-N terminal peptide serum (■), LR1 anti-whole molecule serum (△), or NRS (□) were heat inactivated and then added at 1:20 dilution to Vpr-containing wells. ●, Vpr. (*C*) Titration of Vpr activity demonstrates dose-responsive nature of Vpr activation. Fold dilution: 5 (●), 10 (△), 25 (▲), 50 (○); no Vpr, ■. (*D*) Failure of anti-Vpr serum (LR1) to inhibit phorbol 12-myristate 13-acetate (PMA, 50 ng/ml)-induced activation of HIV expression demonstrates that antibody inhibition is specific for the extracellular Vpr molecule. △, PMA plus LR1; ■, PMA; ○, control.

isolated through nonspecific interactions with the column matrix. In the HIV$^+$ serum, a major band at 15 kDa was observed, corresponding in size to Vpr. The fractions containing this band reacted with anti-Vpr antibodies in ELISA (data not shown). In fraction three, several minor bands were observed at 10–17 kDa. These reacted with anti-Vpr antibodies but did not appear on a second purification of Vpr from the same samples, and so may represent proteolysis fragments and oligomers. These bands were not characterized further. The process of isolation of Vpr removed at least 90% of the Vpr present in the serum (data not shown). No infectious virus remained after this procedure.

Serum Vpr Activates HIV Expression from Latently Infected Cell Lines. Five latently infected cell lines developed in other laboratories were exposed to serum Vpr, and the presence of viral antigen in the culture medium was measured. In each case, serum Vpr activated virus expression within 24 hr, and expression continued for at least 5 days (Fig. 3 *A* and *E–H*). In a further experiment, HIV expression was examined in U1, J1.1, and ACH-2 cells for 2 weeks, and HIV expression was observed throughout this time following a single exposure to Vpr (data not shown). The column eluate from HIV$^-$ human serum, prepared identically to the HIV$^+$ eluate (Vpr), failed to activate HIV expression from these lines.

The Vpr activity was dose-dependent (Fig. 3*C*), with significant activity observed for very low levels of serum Vpr (at least 50-fold dilution from serum levels). The main difference between the activities of the two highest doses of Vpr was found in the kinetics of HIV replication and not in the highest level of virus expression reached. However, with a <10-fold dilution of Vpr, a reduction in the peak expression was observed without a further delay in peak expression. This result suggests that there is a bimodal activity, with level of expression and kinetics being separable.

To further demonstrate the Vpr specificity of this effect, anti-Vpr peptide serum (808), anti-Vpr protein serum (LR1), or nonimmune rabbit serum was included with Vpr in some wells of the ACH-2 line. Both of the anti-Vpr sera inhibited Vpr activity, whereas the nonimmune serum had no effect (Fig. 3*B*). Anti-Vpr antibodies had no effect on PMA activation of HIV expression (Fig. 3*D*); therefore, anti-Vpr antibodies do not generally affect virus expression. These results demonstrate that the activation of HIV expression was the result of Vpr, and they suggest that humoral immunity modulates Vpr activity.

Serum Vpr Activates HIV Expression from PBMCs of HIV$^+$ Individuals. To extend the significance of the Vpr activity, we isolated PBMCs from three HIV$^+$ individuals and examined virus production in the presence of either medium alone, PMA, the mitogen phytohemagglutinin (PHA), or purified serum Vpr (Fig. 4). In the absence of stimulation, HIV-infected PBMCs failed to produce significant levels of HIV antigens. On the other hand, PHA and PMA activated HIV expression from each patient's PBMCs within 1 day, and expression peaked within 2 or 3 days following exposure. Serum Vpr activated HIV expression within 1 day following exposure, and virus expression continued for several days, with the peak production being at day 3 or 4. Peak supernatant p24 levels in the Vpr cultures were 1.5–3 times those in the PHA and PMA cultures.

DISCUSSION

Cytokine activation of HIV is well documented for latently infected cell lines or PBMCs of HIV-infected patients (reviewed in refs. 1 and 13). The U1 cell line, derived from the promonocytic line U-937, is the best studied latent system. HIV expression from U1 may be induced by interleukin 6, interleukin 1, interferon γ, tumor necrosis factor α, granulocyte/macrophage-colony-stimulating factor, PMA, or heat (12, 15, 20, 38, 39). Unstimulated U1 cells express very little

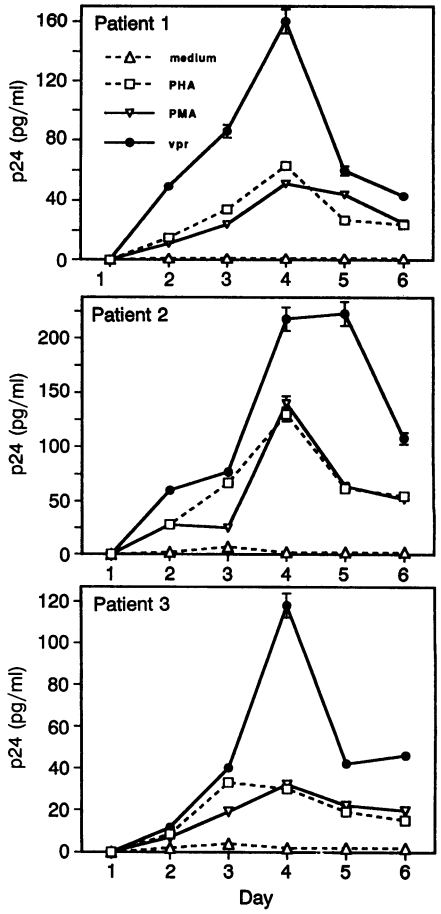

FIG. 4. Induction of HIV replication in resting PBMCs of HIV$^+$ individuals by purified serum Vpr. Medium only (△), Vpr at 0.2 times the serum concentration (●), PHA (5 μg/ml) (□), or PMA (50 ng/ml) (▽) was incubated with 4×10^4 freshly isolated PBMCs on day 1 in duplicate wells of a 96-well plate. Vpr was added also on day 2.

viral message, and the message that is expressed is exclusively fully spliced, with no mRNA for structural proteins (14). Stimulation leads to increased levels of singly spliced and unspliced HIV message and to virus production (14). The ability of a viral protein, Vpr, to induce HIV expression in a manner analogous to cytokines or hormones provides a simple and direct means of activating virus in a viral autocrine or paracrine fashion. The lymphoid tissues are regions in which a relatively high number of cells are infected with HIV in close proximity to each other (2–4, 40). Foci of replication, for example germinal centers, would be likely areas of Vpr enhancement of HIV replication as a result of locally high concentrations of viral antigens.

Clearly, there is sufficient Vpr present in the blood to activate HIV expression *in vivo*, yet activation of virus expression from PBMCs requires *in vitro* stimulation. Most of the Vpr in the serum is probably bound either by viral proteins—e.g., the Gag proteins, which have been shown to be required for virion incorporation of Vpr (41, 42)—or by anti-Vpr antibodies. Anti-Vpr antibodies are present in a significant proportion of HIV-infected individuals (43–45). Therefore, our process of Vpr purification from serum removed the inhibitors of Vpr activity. We observed neutralization of Vpr activity by anti-Vpr antibodies *in vitro*, suggesting that such a mechanism is likely *in vivo*.

The simplest and most probable mechanism through which Vpr may increase HIV expression is by direct or indirect activation of proviral transcription. A weak transactive activity has been reported following transfection of the *vpr* gene

into cells containing reporter plasmids (46), though the levels of HIV transcriptional activation observed were probably too small to account for the great increases in viral expression we find with Vpr protein. It may be that Vpr is more active in a soluble, cell- and virus-free form than it is when expressed endogenously. Therefore, the primary activity of Vpr may be seen in serum protein. Though we cannot rule out the possibility that Vpr could also operate through posttranscriptional mechanisms, a transactive function would be consistent with our previous observation that Vpr activates cellular genetic programs which determine proliferation and differentiation potential (28). We have observed Vpr effects in a wide variety of cell types, including muscle, bone, T and B lymphoid, and myeloid, both transformed and untransformed (28, 47, 48). Therefore Vpr targets a fundamental cellular pathway(s) common to many cell types. Since Vpr affects cell growth and differentiation, serum Vpr might contribute to the diverse pathologies associated with HIV infection, such as neurological disease and immune deficiency. Abnormal development of immune precursors would lead to a gradual decline in mature effector populations and loss of immune function.

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